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PHOTOACOUSTIC MEASUREMENTS OF PHOTOSYNTHETIC ACTIVITIES IN WHOLE LEAVES

PHOTOCHEMISTRY AND GAS EXCHANGE

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Photosynthetic activities of sections of intact tobacco leaves (*Nicotina tabacum* L. var. Xanthi) were monitored by the photoacoustic effect. A reference signal was obtained using brief simultaneous illumination of the leaf with continuous light of saturating intensity. 1. The effect of the direct continuous light depends on the modulation frequency: A decrease in the photoacoustic signal, 'negative effect', is observed at low frequency (under approx. 200 Hz) and an increase of the photoacoustic signal, 'positive effect', at high frequency (above approx. 200 Hz). Both effects are reversible. 2. When leaves are water-infiltrated only a 'positive effect': of the direct continuous light is observed, at any frequency. Leaves infiltrated with aqueous DCMU solution (50 μM) do not show any direct continuous light effect. 3. The 'negative effect' develops, as the leaf is illuminated, in parallel to the (low frequency) photoacoustic signal itself which increases gradually after an initial lag period. This transient phenomenon takes a few minutes under our conditions. The differential increase of the signal during the induction period matches the size of the negative effect obtained by applying the direct continuous light at various times during the induction or in the steady state. 4. In a similar way, the 'positive effect' increases during the induction period, due to a transient decrease of the photoacoustic signal measured at high frequency. This change in the 'positive effect' is less noticeable, as a sizeable effect is already observed in a dark-adapted leaf, upon illumination, and the extent of the photoacoustic transient is relatively small. 5. No photoacoustic transients are observed when direct continuous light is used continuously. 6. It is concluded that the photoacoustic signal is due to two contributions: At low frequency there is a considerable contribution from modulated oxygen evolution, which tends to zero as the direct continuous light is applied. At high frequency the main contribution is from the conversion of photon energy to heat, and direct continuous light effects reflect mainly photochemical energy storage. Both low and high frequency photoacoustic transients reflect the induction period of photosynthesis. 7. Preliminary calculations on the relative damping of modulated oxygen evolution and thermal photoacoustic contributions with the modulation frequency, are consistent with the above model, taking into account diffusion and the rate-limiting step in O_2 evolution. 8. Photoacoustic data were used to construct a relative quantum yield spectrum for both oxygen evolution and photochemical energy storage.

Introduction

The photoacoustic technique ordinarily involves the detection of modulated heat release resulting from the absorption of intensity modulated light by a given sample [1,2]. The heat, generated by the radiationless decay of excited states, diffuses to the sample surface and causes a small layer of gas near the surface to expand and contract periodically. The acoustic waves thus formed are usually detected by a mi-

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crophone and analyzed by a lock-in amplifier for amplitude and phase. The resulting signal depends on the modulated light intensity, the absorption coefficient, the thermal and elastic properties of the sample and the adjacent gas, as well as on the modulation frequency [1–5].

In the case of a photochemically active sample the photoacoustic signal depends on the relative amount of the photon energy which is stored in the photochemical intermediates during each modulation cycle [6]. Direct information on this energy storage can be obtained when the photoacoustic signal from a photoactive material is compared to a suitable reference with similar optical and thermal characteristics but with no apparent photoactivity (i.e., when either the direct yield of radiationless transition is very nearly 1, or when all the possible photoactivities occur on a timescale much shorter than the modulation cycle and lead back to the ground state). In previous experiments with photosynthetic systems (i.e., chloroplasts, cells of *Rhodosprillum rubrum*) [7,8] an efficient and easy method to obtain such a reference was developed. When background light of constant (non-modulated) and high intensity is added to the modulated light, the photochemistry is saturated (i.e., its yield tends to zero) and practically all of the absorbed modulated light is converted to heat, resulting in a maximal photoacoustic signal. The relative decrease in the photoacoustic signal from an active preparation compared to that of a photochemically saturated one, obtained with background light, was called 'photochemical loss'. The photochemical loss is proportional to the quantum yield times the fraction of the photon energy stored for a unit reaction sequence occurring during the modulation cycle. The dependence of the photochemical loss on the modulation frequency, the wavelength and intensity of the modulated light, and on the intensity of the background light, was investigated in some detail. The results were consistent with the above description.

Previous photoacoustic measurements on leaves usually aimed at straight optical spectroscopy or depth profile analysis of pigments [8–10]. The first investigation of the photochemical aspect was made by Inoue et al. [11] who observed light-induced transients in the photoacoustic signal (at 8.3 Hz) from leaves of *Artemisia lactiflora* and compared them with similar changes in chlorophyll *a* fluorescence, measured simultaneously. The variations in the photoacoustic and fluorescence signals seemed to be approximately complementary but their kinetics did not match exactly. This led to the conclusion that the photoacoustic transients do not directly reflect changes in the photochemical yield as measured by fluorescence. Instead, it was tentatively suggested that the photoacoustic transients reflect light-induced changes in the thermal properties of the leaf during the induction period of photosynthesis [11].

When we applied our method of referencing the photoacoustic signal to leaves, we encountered a new phenomenon at low modulation frequencies: in the steady state (when the transients are over) the signal decreased when strong direct continuous background light was applied [12], contrary to that was found for previously investigated photosynthetic systems in suspensions [7,8]. These experiments as well as experiments reported here led to the conclusion that in leaves the photoacoustic signal is composite, made up of essentially two different contributions: first, the conventional one due to the conversion of modulated heat to modulated pressure, and second, a signal due to pressure modulation directly induced by modulated photosynthetic oxygen evolution. This second contribution is particularly important at low modulation frequencies (approx. 100 Hz and below). It is damped much more than the second as the frequency increases and becomes insignificant at frequencies above about 200 Hz. We will show (see also Ref. 12) that the photoacoustic transients observed in leaves at low frequency reflect changes in this contribution rather than changes in thermal parameters as suggested in Ref. 11. The two contributions are distinguished by their opposite response to background light: the extent of the thermal contribution will increase and that due to oxygen evolution will decrease (actually, it will tend to zero). These two effects are observed, separately, at high and low frequency, respectively.

Here we report the results of a comprehensive study of these effects, which support the above interpretation. The utilization for the study of photosynthesis in intact leaves is exemplified.

Materials and Methods

We used mature tobacco leaves (*Nicotiana tabacum* L. var. Zanthi) for the photoacoustic experi-

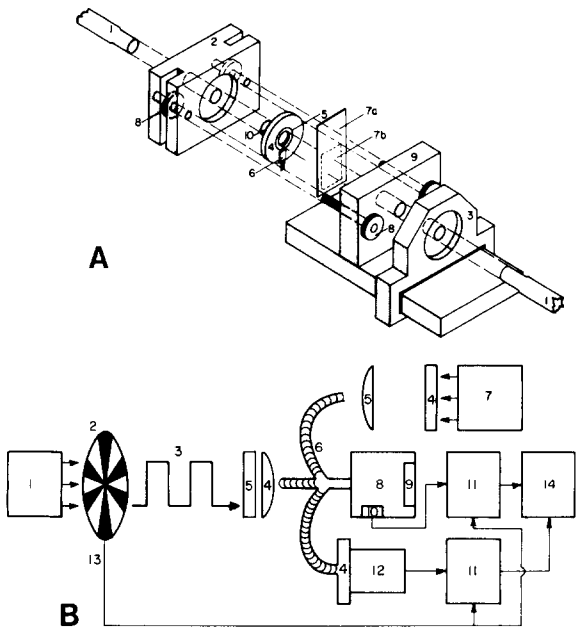

Fig. 1. Illustration of the experimental system. A. Photoacoustic cell. The cell is made of plexiglass, allowing illumination with light guides (1) from both sides (in our case from the left side). All parts are clamped by screws (8) which force the main frame, parts 2 and 9 (and 3) together. A small volume (approx. 90 μl) is enclosed inside ring (4), containing the microphone (6). This core of the photoacoustic cell is sealed by a small rigid light guide (10), an O-ring (5) and the sample itself (7b) which is supported on a glass microscope slide (7a). B. Experimental arrangement. (1) Xe lamp for the modulated light. (2) Chopper. (3) Modulated light (symbolize). (4) Filter(s). (5) Lens(es). (6) Triple-branch homogenized light guide. (6) Filter(s). (7) Light source (projector) operated on d.c. (for background light). (8) Photoacoustic cell. (9) Leaf sample. (10) Microphone. (11) Lock-in amplifiers, for photoacoustic and for fluorescence signals. (12) Photomultiplier, for fluorescence detection. (13) Reference signal from the chopper to synchronize the lock-in amplifiers. (14) Two-pen chart recorder.

ments. The plants were grown in a greenhouse under a 16 h light/8 h dark regime for 3 months. The temperature range was from 18°C (night) to 28°C (day). The leaves used were about 12 cm long. For some experiments sections of leaves were rapidly (approx. 10 s) vacuum-infiltrated with water or an aqueous solution of DCMU (50 μM).

Small sections (2 X 3 cm) were cut and placed in the photoacoustic instrument. The leaf section, supported from behind by a glass microscope slide, formed one wall of the photoacoustic cell (Fig. 1A), described elsewhere [13]. Measurements were carried out with the upper epidermis of the leaf facing the light. The 1 cm diameter leaf disk inside the cell showed no signs of damage, even after prolonged measurements.

The experimental system is shown in Fig. 1B. Light from a 450 W xenon lamp was modulated with a mechanical chopper and passed through a water filter and a narrow-band interference filter. The light was directed onto the sample with a randomized triple light guide (Schott), allowing simultaneous irradiation with the modulated light, usually at 680 nm, and the continuous background light from a direct continuous lamp. The background light was filtered through the standard heat absorber of the slide projector and a water filter, giving a broad band of wavelengths between 400 and 720 nm. In some experiments we used the third branch of the light guide to measure the modulated chlorophyll *a* fluorescence through a 730 nm interference filter (Schott), with a photomultiplier (EMI 9558). In this case, a cut-off filter (less than 660 nm) was placed between the direct continuous projector and the sample. Acoustic and fluorescence signals were processed separately by lock-in amplifiers (Brookdeal 9502 SC and PAR 128) and recorded with a two-pen chart recorder (Houston Instruments). Spectra were recorded using a Bausch and Lomb monochromator (33-86-76 grating, 19.2 nm bandpass) with blocking filters. Light intensities were measured with a YSI-Kettering 65A Radiometer. All measurements were carried out at 21 ± 2°C.

Results and Discussion

Several characteristics of photoacoustic signals from leaves were immediately recognized: (1) occurrence of typical transients, which take usually a few minutes for completion before a steady state is achieved; (2) modulation frequency dependence of the direction, extent and shape of these transients; (3) dependence of the effect of background light on time of application during the transient period; (4) background light effects depending on the modulation frequency.

Frequency dependence of the background light effect

In order to arrive at a reasonable interpretation of the background light effect and its frequency dependence, experiments were first conducted in a steady-state situation. We found large differences between the photoacoustic signal measured with low intensity modulated light only and the photoacoustic signal recorded during simultaneous saturating background direct continuous illumination. Depending on the modulation frequency (f), the signal either decreased or increased upon addition of the background light. Fig. 2 gives results for three typical experiments at different f values, performed with the same leaf disc.

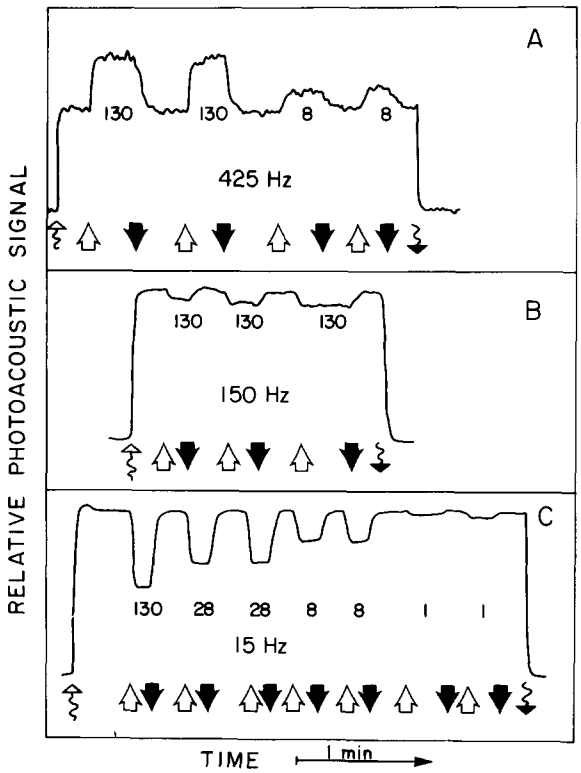

Fig. 2. Effect of background light application on the photoacoustic signal at three modulation frequencies. A: 425 Hz; B: 150 Hz; C: 15 Hz. ⇞, modulated light on, ⇟, modulated light off, ⇧, saturating background light on; ⇩, background light off. The numbers indicate the background light intensity in W/m^2 the highest number nearly corresponds to saturation of the effect. Modulated light: 680 nm, 14 W/m^2. The background light effects were completely reversible within a few seconds when the background light was switched off and could be repeated many times.

Above about 200 Hz the leaf showed the expected phenomenon of 'photochemical loss' (Fig. 2A); i.e., the background light caused an increase in the signal [7,8]. This reflects, presumably, the fact that at these frequencies the signal is essentially due to modulated heat release only (see Introduction). As the frequency was lowered, the apparent relative photochemical loss (in percent of the signal with background light) became smaller until below about 200 Hz a new effect appeared: the background illumination resulted in a decrease of the signal (Fig. 2B). The extent of this phenomenon became progressively more important as f was lowered further, reaching almost half of the total signal (Fig. 2C). For brevity we will call the increase of the signal at high frequencies the positive (abbreviated (+)) effect, and similarly the decrease of the signal at low frequencies the negative (abbreviated (−)) effect.

The (−) effect cannot be explained by photochemical energy storage, unless one assumes that endothermic reactions occur in the time range of about 1–5 ms, which is highly unlikely. A more likely possibility can be suggested if we realize that photosynthesis involves gas uptake and evolution. One would expect the microphone to respond to pressure changes from both modulated heat release and modulated gas production or consumption. Indeed, photoacoustic spectroscopy has been applied to photochemical reactions involving gas uptake, and gas evolution by photocatalytic decomposition [14]; at 60 Hz, pressure changes in the photoacoustic cell corresponding to gas uptake or production of less than a picomole per cycle could be detected.

When considering what type of gas exchange can be sensed photoacoustically it was immediately obvious that the most likely candidate is oxygen evolution. Joliot et al. [15,16] measured modulated oxygen evolution from chloroplasts and algae, using a rate oxygen electrode, up to about 600 Hz. The modulated signal was strongly damped as the frequency increased due to rate-limiting processes, including diffusion. Estimates of the oxygen evolution component in the photoacoustic signal, based on the sensitivity of the cell/microphone combination (Ref. 13, see also Appendix) are in agreement with the observed decrease of the low-frequency signal upon background illumination. According to this calculation, the oxygen evolution component is damped

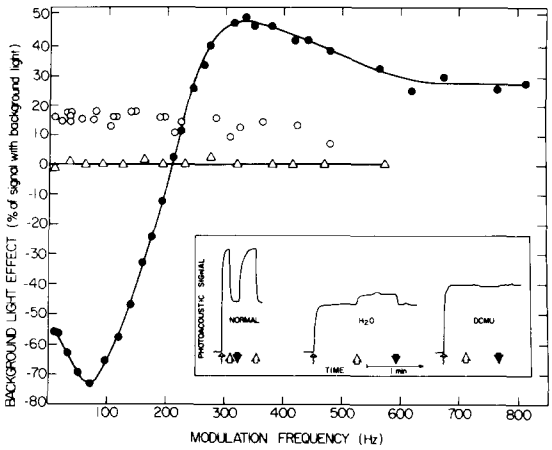

Fig. 3. Effect of background light application as a function of the modulation frequency. Normal (•———•), water-infiltrated (o) and 50 μM DCMU-infiltrated leaves (△———△). Modulated light: 680 nm, 14 W/m^2. Background light intensity: 70 W/m^2. Inset: typical recordings at 35 Hz for (1) normal leaf (2) leaf, vacuum-infiltrated with 0.5% methanol (v/v) in water and (3) leaf infiltrated with 50 μM DCMU in 0.5% methanol, 15 min before the experiment. Symbols as in Fig. 2.

and becomes too small to be detected at high frequencies (see Appendix). Theoretically, it is unlikely that CO_2 uptake contributes to the signal, as its modulation is expected to be considerably damped due to the existence of large pools of intermediates and relatively long reaction times between the photochemical and the CO_2 fixation reactions (see Appendix).

The above interpretation is supported by results from several experiments such as comparative studies on normal leaves and leaves infiltrated with water or with 50 μM DCMU solution. The complete frequency dependence of the background light effect is shown for these three cases in Fig. 3. For leaves infiltrated with water the (−) effect disappeared completely, while the positive photochemical-loss effect persisted and was observed even at low modulation frequencies. This observation is expected: in an infiltrated leaf, water fills the intercellular spaces previously occupied by air and oxygen diffusion from the cells takes place first through this extracellular water. Thus the effective diffusion path to the outer air phase increases from roughly 2 μm – the average chloroplast to cell wall distance – to at least 30 μm – the distance to the leaf surface when water fills the intercellular air space. The diffusion time consequently increases enormously, damping the modulation severely and actually eliminating altogether the O_2 evolution contribution that can be detected photoacoustically. The thermal conversion contribution is also damped, but not as much as the O_2 evolution (see Appendix), and therefore it becomes the sole factor, reflected in the photoacoustic signal. With the elimination of the masking effect of the O_2 evolution the 'normal' photochemical-loss effect is now apparent also at low frequencies. It should be mentioned that the photochemical apparatus by itself is not significantly changed by the infiltration, judged from the presence of an almost normal fluorescence induction pattern (see also Ref. 17).

Infiltration of the leaf with 50 μM DCMU solution, which inhibits photosynthesis in toto, causes complete inhibition of the background light effect, as expected.

Light-intensity dependence of the background light effect

Fig. 2. shows examples of raw data for various direct continuous light intensities. It was previously shown in suspensions of chloroplasts and *R. rubrum* that the (+) effect of the background light tends to saturation as its intensity increases [7,8]. We expect a similar behaviour also in leaves for both the (+) and (−) effects. Indeed, Fig. 4 shows that both effects

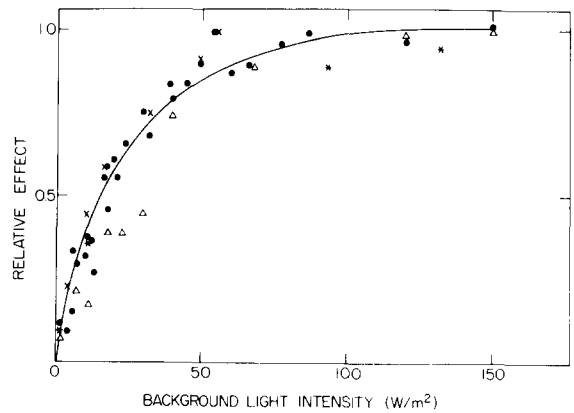

Fig. 4. Background light intensity dependence of the (+) and (−) effects and the fluorescence yield. The changes were normalized to those at background light intensity of 150 W/m^2. •, negative effect, at 35 Hz; *, positive effect at 200 Hz; X, positive effect at 362 Hz; △, fluorescence yield. Modulated light; 680 nm, 14 W/m^2. Background light, $\lambda < 660$ nm.

tend to saturation, with matching light-intensity dependencies. Moreover, the fluorescence yield was also measured simultaneously and showed similar (background) light intensity dependence. This is consistent with our expectations: oxygen evolution and fluorescence are competing processes, arising in Photosystem II [18]. Writing the modulated oxygen evolution as $\phi_{O_2} \cdot \tilde{I}$, where ϕ_{O_2} is the quantum yield and $\tilde{I}$ is the modulated light intensity, the background light dependence of the resulting signal follows the changes in ϕ_{O_2}. Similarly the modulated fluorescence signal, written as $\phi_F \cdot \tilde{I}$, reveals the dependence of ϕ_F on the background light intensity. Both ϕ_{O_2} and ϕ_F are expected to change in a complementary fashion [18], so that the relative increase of the fluorescence should match the relative decrease of the oxygen evolution, as indeed is observed. Our results indicate that both (+) and (–) effect, as well as fluorescence, are manifestations of the same photochemical activity (i.e., PS II).

An alternative way of looking at the light intensity dependence is to examine how the maximal effect of saturating background light decreases, relative to the total photoacoustic signal, as the intensity of the modulated light increases, as demonstrated in Fig. 5 for the (–) effect, and as shown previously [7,8] for the photochemical-loss, (+) effect in chloroplasts and *R. rubrum*. This decrease is due to the fact that with increasing intensity (of the modulated light) the system already moves partially towards photochemical saturation, and its photoacoustic signal approaches that of the referenced state (i.e., complete saturation).

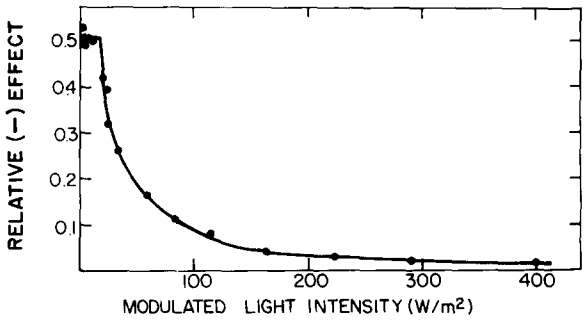

Fig. 5. Decrease in the (–) effect (relative to the total signal with background light) as a function of the modulated light-intensity. In this experiment the light from the Xe lamp was passed through a water filter only. Modulation frequency, 35 Hz: Background light intensity, 80 W/m^2.

Photoacoustic transients and the relation to the background light effect

As observed previously [11,12] the photoacoustic signal changes continuously during the initial period of illumination, when using dark-adapted leaves. At low frequencies (approx. 10–30 Hz) the signal generally increases from a certain initial low value (P_o) to a steady-state value (P_s). The extent of the increase was usually quite considerable: the steady-state signal (P_s) was often more than twice the initial signal (P_o). We did not achieve, however, clear control over the time dependence of this increase. In some cases there was a considerable lag (of many minutes) before the start of the photoacoustic signal increase ('long' transient) while in other cases the photoacoustic increase started almost immediately ('short' transient). Leaves cut from the same plant differed quite a bit in their detailed time response. On the other hand, after once achieving a steady state and allowing a certain dark relaxation period (approx. 8 min) the resulting short transient upon resumption of the illumination was much more reproducible. Shorter dark-adaptation periods did not bring about full restoration of the 'short' transient, as also reported in Ref. 11. Also, quite often, if some small holes were picked delicately with a syringe needle on the side of the leaf facing the photoacoustic chamber, 'short' transients

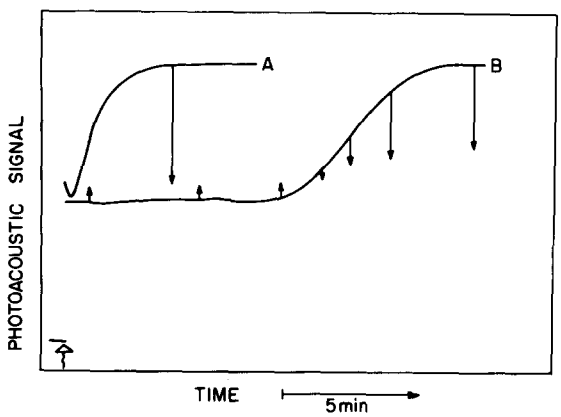

Fig. 6. Two types of photoacoustic transient observed for freshly cut leaves after 8 min dark adaptation. A, 'short transient'. B, long transient. Modulation frequency, 35 Hz. $\hat{\xi}$, modulated light (680 nm, 14 W/m^2) on. Arrows indicate the size and direction of the background light (80 W/m^2) effect at various moments during the transient. (Saturation here is not complete.)

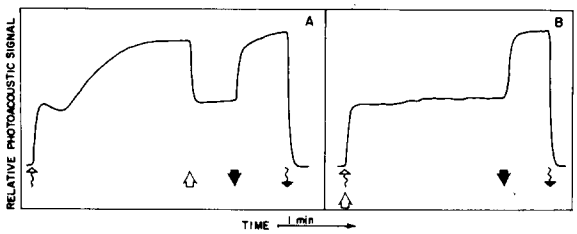

Fig. 7. Low-frequency (35 Hz) photoacoustic transients from dark-adapted leaf and the effect of background light, in the steady state (A) or when applied from the start of the experiment and switched off in the supposed steady-state (B). ⇳, modulated light (680 nm, 14 W/m^2) on. ⇓, modulated light off. ⇑, background light (white, 340 W/m^2) on. ↓, background light off.

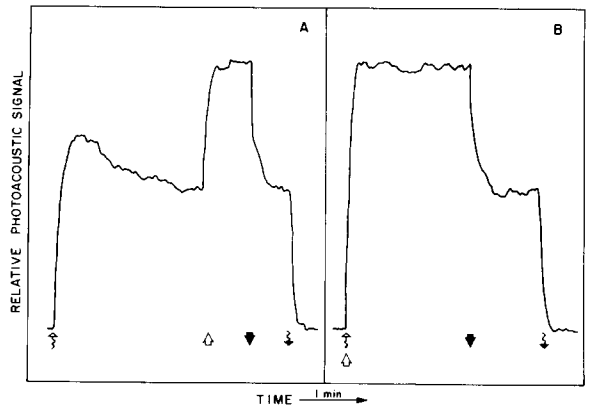

Fig. 8. High frequency (292 Hz) photoacoustic transients from dark-adapted leaf. Same as Fig. 7.

were obtained with a fully dark-adapted leaf. The two typical kinds of transient are shown in Fig. 6. The 'short' transient is similar to that reported by Inoue et al. for *Artemisia lactiflora* [11].

The (−) effect could not be observed at all until the photoacoustic transient had developed. Actually, at the start there was always a (+) effect, even at low frequencies. This was particularly clear in the case of 'long' transients where it was possible to repeat the background light effect several times. As the photoacoustic signal increased progressively in time the (+) effect decreased and gradually the (−) effect developed until it became maximal as the steady photoacoustic signal, P_s, was achieved (Fig. 6).

The connection between the transient and the (−) effect is clearly shown by noting that the (−) effect of the background light brought the signal back close to the initial level P_o (Fig. 7A). If both background light and modulated light were applied from the start, no transient was found. However, if the background light was switched off after an illumination time corresponding to the transient period, the signal immediately increased to the P_s value (Fig. 7B). According to our working hypothesis these observations are explained as follows: The P_o value obtained when illumination starts reflects only thermal conversion; there is essentially no oxygen evolution. Hence, the background light effect is only positive. With time, the oxygen evolution gradually increases and is reflected by the increasing contribution of the corresponding photoacoustic signal. The background light tends to abolish this contribution and a signal close to the P_o level (actually slightly above P_o, due to the (+) effect on the thermal conversion part) is obtained.

The fact that photosynthetic oxygen evolution does not start immediately upon illumination but shows long and complicated induction phenomena has been known for quite some time (for example, see the review by Walker [19]. Indeed, there are aspects of the (low-frequency) photoacoustic transients which are similar to the time course reported for induction of photosynthetic oxygen evolution. The fact that a 'positive effect' is noticed with no oxygen evolution indicates the presence of some other kind of energy-storing photochemical activity (possibly cyclic electron flow for ATP production).

The photoacoustic signal measured at high frequency also undergoes a transient, very similar in its time dependence to the low frequency signal, but in the opposite direction (Fig. 8). Since at high frequency the signal represents only thermal conversion, the signal decrease with time presumably reflects an increase of the photochemical loss (i.e., increasing photochemical activity). Application of saturating background light during various times of the transient brings the signal always to the same (higher) level, above the initial value P_o, which represents full thermal conversion.

In their work, Inoue et al. [11] suggested another explanation for the (low frequency) photoacoustic transients, being a reflection of light-induced changes in the thermal diffusion parameters of the leaf. Their hypothesis was based on the known dependence of the photoacoustic signal on such a parameter [1]. But

the fact that most of the thermal resistance in leaves is due to the water phase between photosynthetic membranes within the cell, and to the outer envelope of the cell facing the air phase, argues against this suggestion. One needs to postulate considerable changes in the cell volume or shape during the $P_o \rightarrow P_s$ transition to account for such a major change in the thermal parameters. Alternatively a movement of the plastids closer to the cell outer membrane has to be assumed. Such changes must be induced by massive water transport during the transient time and it seems very unlikely that such a process really occurs. Our experimental results argue more specifically against such an interpretation. First, with background light on from the start of modulated illumination, there is no transient at all. The photoacoustic level remains close to P_o. If at the end of a time period corresponding to the transient time, the background light is switched off, the photoacoustic signal changes immediately (limited by the response time of the instrument: approx. 1 s) to the P_s level (see Fig. 7). One expects that the background light by itself should not interfere with the postulated light-induced changes in the thermal parameters. Rather it should enhance them. Therefore, such changes should still be reflected in corresponding variations in the photoacoustic signal even when background light is present. Furthermore, even in the unlikely situation that somehow the background light inhibits the process inducing the change in thermal parameters, the time actually observed for the $P_o \rightarrow P_s$ transition upon switching off the background light is indeed too short compared to the photoacoustic induction time in modulated light, and compared to what one expects for the time of water transport through the cell envelope (10^2–10^4 s [19]). Moreover, according to this hypothesis, the photoacoustic changes during the induction period should always have the same direction, no matter what modulation frequency is used, which is in contradiction with the experiment (Figs. 7 and 8).

Actually, since in the presence of saturating background light and hence full thermal conversion, the photoacoustic signal remains perfectly stable, one can conclude that the crucial anatomical parameters (geometry, distances, etc.) of the leaf do not change at all during illumination.

Possible separation of the oxygen-evolution and thermal-conversion contributions

We conclude that the photoacoustic signal from leaves is composed, in general, of two main contributions; $P^{(O_2)}$, representing oxygen evolution, and P^{th} representing the thermal conversion contribution. The latter contribution is made up of P^{th}_{max}–(PL) in which P^{th}_{max} corresponds to maximum thermal conversion of the photon and (PL) is the 'photochemical loss' due to photochemical energy storage. To distinguish between these contributions, direct continuous background light is used and the signal in this case will be denoted by P_{bkg}. Hence, in general:

$$P = P^{(O_2)} + P^{th}_{max} - (PL) \tag{1}$$

$$P_{(bkg)} = P^{th}_{max} \tag{2}$$

and therefore

$$P - P_{(bkg)} = P^{(O_2)} - (PL) \tag{3}$$

The parameter $P_{(bkg)}$ is time-independent, as is obvious (see Figs. 7 and 8). Assuming that at time zero of the transient there is no modulated oxygen evolution (particularly in the 'long' transients), we may write for P_o and $(PL)_o$, the initial values of P and (PL), respectively:

$$P_o = P^{th}_{max} - (PL)_o = P_{(bkg)} - (PL)_o \tag{4}$$

or

$$(PL)_o = P_{(bkg)} - P_o$$

The last relation could be used to estimate $P^{(O_2)}$ in Eqn. 3 provided that the photochemical loss remains constant, equal to $(PL)_o$, during the transient. However, since the photochemical loss presumably also changes during the transient, we require more information to estimate its change. It is plausible that the change in photochemical loss comes mainly from the change in the quantum efficiency of the photochemistry and, therefore, the change of photochemical loss, relative to its initial value $(PL)_o$, should be the same at all frequencies. At very high frequency the O_2 contribution is damped so much that it can be neglected. Denoting photoacoustic mea-

surement at such a high frequency by $\tilde{P}$ one therefore obtains:

$$\tilde{P}_{(bkg)} - \tilde{P} = (\tilde{PL}) \quad \text{(from Eqn. 3)} \tag{5}$$

and

$$(PL) = (PL)_o \cdot \frac{(\tilde{PL})}{(\tilde{PL})_o} \tag{6}$$

and therefore, by using Eqns. 3 and 4

$$P^{(O_2)} = P - P_{(bkg)} + (PL)_o \frac{(\tilde{PL})}{(\tilde{PL})_o}$$

$$= P - P_{(bkg)} + \frac{(P_{(bkg)} - P_o)(\tilde{P}_{(bkg)} - \tilde{P})}{\tilde{P}_{(bkg)} - \tilde{P}_o} \tag{7}$$

All of the quantities in the right-hand side of Eqn. 7 can be read from data such as those in Figs. 7 and 8.

At the lowest frequency the main contribution comes from $P - P_{bkg}$, the third term (=(PL)) being relatively small. In Fig. 9 the computed oxygen evolution contribution to the signal, according to the last equations, is shown together with a normalized transient for O_2 evolution from P_o to P_s. The two are quite similar.

The photoacoustic transient at low frequency often started with a small phase of decrease from P_o to a certain minimum value below P_o and only then started to rise gradually to P_s (Fig. 7). During this decrease, application of background light resulted in a positive effect. Hence, this phase of the transient is characterized by increasing photochemical efficiency, although this is not expressed yet as O_2 evolution. The subsequent rise reflects the onset of the O_2 evolution.

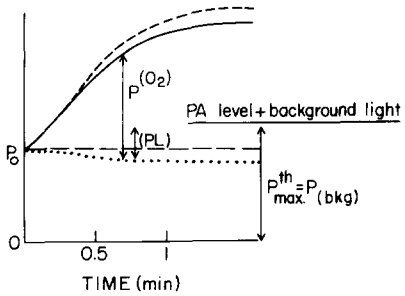

Fig. 9. Evaluation of the different contributions to the photoacoustic signal at low frequency (see text). In this example only the rising part of the transient where O_2 evolution starts is shown. Solid curve, photoacoustic transient at 35 Hz. Dotted curve, calculated $P^{th}_{max} - (PL)$ from data at high frequency and the photoacoustic level with background light, as explained in the text. Dashed curve $P^{(O_2)}$ measured from a baseline at P_0, estimated from the difference between the solid and the dotted curves.

Quantum yield spectra

We used both the (–) effect at 35 Hz and the photochemical loss (+) effect) at 275 Hz to measure the (relative) quantum yield spectrum of the leaf photosynthetic activity (Fig. 10). The wavelength dependent light intensity variations are accounted for by the use of the photoacoustic signal in the presence of background light as a measure of the intensity of the absorbed light. As this signal is proportional to the intensity in power units (i.e., energy/time) it should be multiplied by the wavelength λ, to give a result proportional to the number of absorbed quanta. Thus the ratio magnitude of the (–) effect/photoacoustic signal with background light, divided by λ, is proportional to the quantum yield of oxygen evolution. The other ratio magnitude of the (+) effect/photoacoustic signal with background light, indicates the fraction of energy out of the total incident photon energy which is stored by the photochemical reaction at a given modulation frequency. Multiplying by the energy of the photon (i.e., dividing by λ) yields a number proportional to the quantum yield. The results were nor-

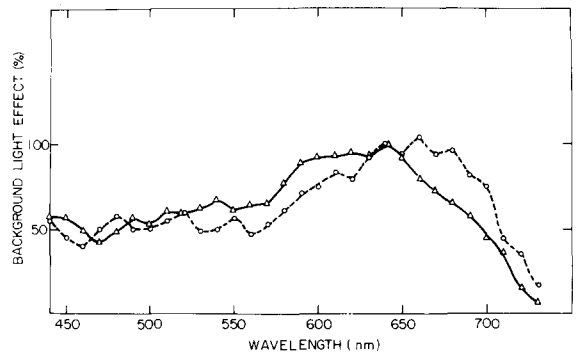

Fig. 10. Quantum yield spectra of photosynthesis from photoacoustic measurements of the (+) and (–) effects. Relative effects corrected for the change in the photon energy vs, wavelength (see text) are plotted vs. the wavelength of the modulated light. Each spectrum is the average of two replicate spectra. All data normalized to 640 nm.

malized to 1 at 650 nm. The Emerson 'red-drop' effect [21] is seen at the far-red side, $\lambda > 680$. Fig. 10 thus demonstrates the utility of the photoacoustic method for rapid scanning of photoactivity vs. wavelength in general and in leaves in particular, where such measurement by any other method is extremely difficult.

The quantum-yield spectra of Fig. 10 are similar, and the differences may be due to arbitrary variations in the sample. However, there may be real differences. For example, the 'red-drop' in the photochemical loss spectrum is less noticeable and perhaps indicates a contribution of photoactivity to Photosystem I alone. It is interesting also that the decline in the O_2 evolution starts at a shorter wavelength (approx. 660 nm) compared to where the usual red drop starts in the Hill reaction [22] or in *Chlorella* [21] (approx. 690 nm). These aspects are being checked again with a better spectral resolution.

Possibility that photoacoustics probes the 'inside' of the leaf, for the photosynthetic oxygen evolution

Experiments in which the leaf was coated with vacuum grease to avoid oxygen diffusion out (and CO_2 uptake) did not inhibit to any significant degree the photoacoustic responses described above (see also Ref. 11). This apparent problem was partially answered by doing experiments in which copper and glass plates were placed between the leaf and the photoacoustic cell (illumination was provided from the back). First we thought that by insertion of a 1 mm copper plate we would eliminate the gas pressure modulation due to O_2 evolution and remain only with the thermal contribution. This was not the case, as both contributions remained in the same proportion although the total signal was attenuated. The same results were obtained with a 2 mm glass slide, which is a thermal insulator. The conclusion was that the conversion to acoustic signal occurs already in the inside of the leaf and the plates serve as transmitters of the mechanical vibrations from the leaf (see Refs. 13, 22). It is therefore conceivable that the outer layer of the leaf (epidermis) is sufficiently elastic to serve as such a transmitter and that what is detected is the modulated oxygen evolution and photothermal conversion inside the leaf. Thus, even if oxygen diffusion out of the leaf is inhibited, the reactions inside the leaf are still detectable.

Conclusions

In this report we have concentrated on characterization of the photoacoustic signals that can be obtained from whole leaves. The salient feature is the sensitivity of the signal to O_2 evolution, presented here still as a working hypothesis. However, recent experiments using photothermal radiometry [24], where only thermal changes are measured by sensitive infrared detection, provide strong direct evidence for this hypothesis, as no (–) effects upon application of direct continuous background light were observed, but rather (+) effects (Bults, Nordal, Kanstad, unpublished data). Therefore we feel that the photoacoustic method, in some cases in conjunction with fluorescence and photothermal radiometric ones, can now be applied to study a variety of basic and applied problems in plant photosynthesis. These may include comparisons between photosynthetic processes in leaves and chloroplast suspensions in general, study of the control exerted by the CO_2 fixation system on the primary reactions, study of the effects of water stress and other ambient conditions, to name but a few. Some such experiments are now underway in our laboratory.

Appendix – calculation of the photoacoustic signal

1. Introduction

A simplified analysis of the relation between modulated gas evolution (or uptake – considered as negative evolution) and the photoacoustic signal is attempted, as a first approach, and will be outlined. The model is taken as unidimensional, consisting of a source (chloroplasts) located at a fixed distance (l) from the boundary separating the aqueous and gaseous phases. More realistic (and much more complex) models do not seem appropriate or practical at this stage of our understanding. The simple model contains, however, all the ingredients essential to the explanation of the experimental phenomena.

If the source is excited with (sinusoidally) modulated light, photosynthetic primary reactions are modulated at the same frequency, and a final product Z (e.g., O_2 or CO_2) is formed at a rate which is also partly modulated. The modulation amplitude for Z is damped compared to that of the primary reaction for two main reasons: (1) the occurrence of a time

lag elapsing between the primary reaction and the formation of Z; (2) the diffusion time for Z from the source to the boundary.

2. Damping due to limiting processes between the primary reactions and formation of Z

The reactions that lead to oxygen, ATP or NADPH production are approximated by the following sequence of first-order steps:

$$A \xrightarrow{h\nu} B_1 \quad B_1 \xrightarrow{k_1} B_2 \quad B_2 \xrightarrow{k_2} B_3 \cdots B_i \xrightarrow{k_i} Z \tag{A1}$$

These lead to the following set of differential equations for the concentrations

$$\frac{dB_1}{dt} = \frac{\phi I}{N} - k_1 B_1 , \quad \frac{dB_2}{dt} = k_1 B_1 - k_2 B_2 ,$$

$$\frac{dB_i}{dt} = k_{i-1} B_{i-1} - k_i B_i \tag{A2}$$

and finally

$$\frac{dZ}{dt} = k_i B_i \tag{A3}$$

where I is the input of (absorbed) light per cross-section of the photoacoustic cell, N is the volume of the photochemical sources (chloroplasts) per cross-section and ϕ is the efficiency and Z the amount of Z formed. This set is easily solved in steps: first for B_1, then B_2, B_3 etc. Finally dZ/dt is expressed through the solution of B_i. For light which is modulated ($I = \bar{I} + \tilde{I} \sin \omega t$) one finally obtains the modulated part of the total rate of generation of Z ($= N\frac{dZ}{dt}$)

$$N\frac{dZ}{dt} = \phi \tilde{I} \cdot \frac{k_1}{\sqrt{k_1^2 + \omega^2}} \cdot \frac{k_2}{\sqrt{k_2^2 + \omega^2}}$$

$$\cdot \frac{k_i}{\sqrt{k_i^2 + \omega^2}} \cos(\omega t - \delta) \tag{A4}$$

where δ is the total phase shift term, having an additive contribution from each step ($\delta \to 0$ when $\omega \to 0$), and the amplitude is attenuated by multiplicative factors of the form $k_r/(k_r^2 + \omega^2)^{1/2}$ for each reaction step. These attenuation factors approach 1 as far as $\omega \ll k_r$.

Limiting steps in the electron transfer leading from Photosystem II to oxygen evolution have rate constants of the order of $1000\ s^{-1}$ [15,16]. This gives an idea of the possible range of frequencies where damping may occur: for a frequency of 10 Hz the attenuation factor is about 1 (actually 0.998). For frequencies near 100 Hz the attenuation factor is about 0.86; 50% attenuation will be reached when $\omega = 270$ Hz.

An analysis of the CO_2 fixation system in terms of its reaction sequence is prohibitively complex due to complicated kinetic expressions, enzymatic mechanism requiring participation of several molecules, branching steps, regulation of the enzymes by the participating molecules and the uncertain information presently available on the in vivo levels of enzymes and substrates under conditions similar to ours. Nevertheless, some estimations will be given to confirm that modulated CO_2 uptake is heavily damped.

Molecules produced in the light reactions (i.e., ATP, NADPH) serve to transmit the modulation to the Calvin cycle intermediates and hence cause, in principle, modulation in the CO_2 uptake. A crucial step, which could possibly affect CO_2 uptake modulation is the phosphorylation of ribulose 5-phosphate by ATP, just prior to the carboxylation. The steady-state level of ATP is estimated to be around 1–2 mM [25] 2–4-times above the K_m value for ATP of ribulose-5-phosphate kinase [26]. A regular light intensity of the order of approx. $10\ nE \cdot cm^{-3} \cdot s^{-1}$, modulated with angular frequency ω, can cause, at most, a modulation in the ATP level of amplitude $(1/\omega)$ of about 10 μM which is of the order of 1% ($\times\ 1/\omega$) of the ATP level. The modulation amplitude in the rate of the phosphorylation cannot be more than 1% ($\times\ 1/\omega$) and possibly it is much less due to the fact that ATP exists in near saturating concentrations for the enzyme.

Similar considerations exist for the step involving the 3-phosphoglycerate kinase [27]. Moreover, this reaction is the starting point for the chain of reactions for which carboxylation is the final step, and each step in the chain will further damp the modulation. When a substrate is present at saturating levels the modulation is damped due to the fact that changes in its concentration will not affect the enzymatic rates. Taking the less optimal point of view for each reaction step, i.e., that the substrate level is in the linear range of enzymatic activity, it is evident that each step is analogous to a first-order step such

as formulated in Eqn. 4, the first order constant (k_i) being the ratio of the saturation rate of the enzymatic activity (v_{max}) divided by the affinity constant (K_m) of the particular substrate [28]. There are at least two steps with $k_i \approx 10\ s^{-1}$ (i.e., those involving glycerol-3-phosphate dehydrogenase and FDPase) which even at as low a frequency as 10 Hz should together damp the modulation to about 3% (for this information we consulted Refs. 29–31). The modulation of the transmitter NADPH itself is damped initially by a rate-limiting step of about $30\ s^{-1}$ (from typical values of electron transport rates), corresponding to damping of about 0.5 at 10 Hz. The steady level of NADPH is of the order of 0.5–2 mM [32] and the amplitude of its modulation (in similarity to the case of ATP discussed above) is in the order of $1\%(\times 1/\omega)$. Thus, the total modulation of CO_2 uptake must be in the order of $1\%(\times 1/\omega)$, even at the low frequency of 10 Hz, and probably is even much less.

3. Damping by diffusion

This mechanism of damping is similar to that occurring for heat diffusion in photoacoustic signal generation [1,3,5], with differences in some details. The simplest unidimensional model is to represent the chloroplasts as thin elongated layers generating the compound Z, and having their larger cross-section perpendicular to the diffusion path (represented by the variable x). One obtains the usual diffusion equations.

$$\frac{\partial[Z]}{\partial t} = D\frac{\partial^2[Z]}{\partial x^2} \tag{A5}$$

and

$$j = -D\frac{\partial[Z]}{\partial x} \tag{A6}$$

where j is the flux of Z per unit area per unit time. In the steady state the flux at $x = 0$ (i.e., emanating from the source) is approximately equal to the rate of generation of Z per surface area enclosing the source (if the width of the source is kept small, and at relatively low frequencies). This is equal to dZ/dt of Eqns. A3 and A4, multiplied by the volume of the source, divided by its area.

The modulated part of [Z] (the diffusion wave), which is the solution of Eqn. A5 is a mathematical superposition of a primary forward-travelling wave (emanating from the source and travelling to the boundary between the aqueous and gaseous phases) and a wave reflected at the boundary, travelling in the reverse direction. In the gaseous phase there is a (forward-going) transmitted wave. The general form of the forward-travelling primary wave, consistent with the diffusion equation (Eqn. A5) is:

$$[Z] = [\tilde{Z}]_0 \cdot e^{-\sqrt{\omega/2D}\cdot x} \cos\ \omega t - \sqrt{\frac{\omega}{2D}}\cdot x - \delta \tag{A7}$$

The boundary conditions imply a certain relation between the amplitude and phase of the primary wave and those of the reflected and transmitted waves. The conditions usually set are that the material fluxes, j and j_g, and the activities [Z] and $K[Z_g]$, are equal at both sides (subscript g denotes gas parameters, K is the equilibrium partition coefficient between the concentration in the gas and aqueous solution ($K = [Z]/[Z_g]$). It is possible to show, from $j = j_g$ and $[Z] = K[Z_g]$ at the boundary coordinates ($x = l$), that the transmission coefficient for the transmitted wave is:

$$2\sqrt{\frac{D}{D_g}}\quad K\cdot\sqrt{\frac{D}{D_g}} + 1$$

Since $K \approx 2\cdot 10^{-2}$ and $\sqrt{D/D_g} \approx 7\cdot 10^{-4}$ for oxygen, one can approximate the transmission coefficient by $2\sqrt{D/D_g}$ ($\approx 1.4\cdot 10^{-3}$). The transmitted wave is very small and essentially most of the primary wave is reflected. The transmitted wave can be written as

$$[Z_g] = [\tilde{Z}_g]\, e^{-\sqrt{(\omega/2D_g)}\,x_g}$$

$$\times \cos\left(\omega t - \sqrt{\frac{\omega}{2D_g}}\cdot x_g - \sqrt{\frac{\omega}{2D}}\cdot l + \delta\right) \tag{A8}$$

$$x_g \geqslant 0.$$

where x_g is equal to $x - l$, i.e., the distance in the gaseous phase. Eqn. 8 is similar and adjusted in phase to Eqn. A7 at $x = l$ (i.e., $x_g = 0$). The amplitude $[\tilde{Z}_g]$ is written as:

$$[\tilde{Z}_g] = 2\sqrt{\frac{D}{D_g}}\cdot[\tilde{Z}]_0\, e^{-\sqrt{(\omega/2D)}\,l} \tag{A9}$$

4. Calculation of the photoacoustic signal

One can now use the acoustic piston model of Rosencwaig and Gersho [1,3], with the modification

that the piston is activated not by thermal modulation but by concentration modulation. Also, instead of using the gas phase distance l_g, it is more appropriate here to use the ratio V/A where A is the cross-sectional area of the source and V the total gas volume. One obtains for the amplitude of the pressure variation:

$$\delta[\tilde{P}] = \frac{\gamma P_0 A[\tilde{Z}_g]}{\sqrt{2}\, V \sqrt{\frac{\omega}{2D_g}}\, C_0} \tag{A10}$$

where γ is the ratio of the gas specific heats ($= C_p/C_v$) and P_0 and C_0 are the ambient pressure and average total concentration. $[\tilde{Z}_g]$ will now be related to the actual flux at $x = 0$ (i.e., at the source). This is obtained from Eqn. A7, using Eqn. A6. In calculating the flux and comparing to that generated by the light reactions one has to consider: (a) that the total net flux at $x = 0$ due to the primary wave is twice that obtained by differentiation of Eqn. 7 (as the source has two sides, while only one diffusion direction, corresponding to the closest chloroplast-cell wall distance, actually contributes to the final output signal); (b) The net flux of the reflected diffusion wave at $x = 0$ is largely eliminated as the fluxes at each side of the source are almost equal and therefore cancel each other. Thus the amplitude of the total flux at the source is:

$$\tilde{F} = 2\tilde{j}_0 = 2 \cdot \sqrt{\frac{\omega}{2D}} \cdot D[\tilde{Z}]_0 = \sqrt{2\omega D} \cdot [\tilde{Z}]_0 \tag{A11}$$

using Eqns. A9–A11, one arrives finally at:

$$\delta[\tilde{P}] = \frac{\gamma P_0 \cdot \tilde{F}A}{\omega \cdot V \cdot C_0} \cdot e^{-\sqrt{(\omega/2D)} \cdot l} \tag{A12}$$

Summing $\tilde{F}A$ over all the contributing sources ($\Sigma \tilde{F}A$) will give the amplitude of the total input rate of Z generation (see Eqn. A4). The resulting final total pressure modulation amplitudes will be:

$$[\tilde{P}] = \frac{\gamma P_0}{\omega V C_0} \cdot e^{-\sqrt{(\omega/2D)} \cdot l} \times \left(\prod_i \frac{k_i}{\sqrt{k_i^2 + \omega^2}} \right) \phi \cdot \tilde{I} \tag{A13}$$

Summing up, the photoacoustic signal due to gas evolution is made up of the following terms:

(a) A numerical factor, $\gamma \cdot P_0/C_0 V$, which for ordinary situations (ambient pressure = 1 bar, $C_0 = 4.5 \cdot 10^{-5}$ mol/cm^3), our cell volume of about 0.1 cm^3, is approximately equal to $3 \cdot 10^4$ bar/mol).

(b) A factor, $1/\omega$, which expresses the frequency dependence when no damping occurs.

(c) A factor $\exp -\sqrt{\omega/2D} \cdot l$, which expresses the damping due to diffusion in the aqueous phase.

(d) A multiplicity of factors of the form $k_i/\sqrt{k_i^2 + \omega^2}$, which express the damping due to dark reaction steps which lead to the product.

(e) The light flux per cross section multiplied by its quantum efficiency.

5. Preliminary comparison with the experiment

Basically, a $1/\omega$ dependence was observed for both oxygen evolution and the thermal signal in a range of frequencies, from the lowest (60 rad/s) to about 470 rad/s (i.e., no damping in this ω range). For this range, and for ordinary light intensities of approx. 10^{-8} E/s per cell cross-section, assuming an optimal efficiency $(O_2/h\nu)\phi \approx 0.1$, one obtains for $[\tilde{P}]$

$$[\tilde{P}] \approx 1/\omega \cdot 3 \cdot 10^4 \cdot 0.1 \cdot 10^{-8}$$

e.g. for $\omega \approx 60$ rad/s one obtains $[\tilde{P}] = 0.5$ μbar. Our microphone/amplifier sensitivity is approx. 40 mV/μbar, which should result in a signal of 20 mV. This number is indeed within the range of signal strengths usually obtained (5–20 mV) for the O_2 evolution signal. This correspondence gives us confidence in this model.

The diffusion constant D is in the range of $2 \cdot 10^{-5}$ cm^2/s, which is much smaller than the analogous thermal-diffusivity for the heat conduction approx. $1.5 \cdot 10^{-3}$ cm^2/s. Thus there will be damping in the oxygen signal beginning at lower frequencies compared to the thermal signal. Estimating $l \approx 10^{-4}$ cm this leads to a 'half damping' frequency of approx. 450 Hz for the O_2 signal. This seems quite a high frequency as the O_2 signal is damped quite heavily already at about 200 Hz. It is perhaps possible that the main damping factor is due to the reaction steps, as described in Eqns. A1–4. The sharp frequency-dependence decrease in a rather narrow range of frequencies (between approx. 100 and 250 Hz) may indicate the occurrence of several such steps with similar time constants.

In infiltrated leaves the average distance to be travelled by diffusion is around $30 \cdot 10^{-4}$ cm and the diffusion damping factor for oxygen becomes vanishingly small (approx. 0.01) even for as low a frequency as 10 Hz. The damping factor for the thermal signal is only 0.6, consistent with the results of Fig. 3.

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